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(54) Title: LIPOXYGENASE

(57) Abstract: The inventors have found a novel fungal lipoxygenase from *Magnaporthe salvinii* and determined its sequence. They have sequenced the gene and cloned it into *E. coli* and deposited the clone. Oligonucleotide probes based on the sequence information are useful for screening a eukaryotic library to obtain a lipoxygenase. The lipoxygenase is useful in baking and in a detergent.



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## LIPOXYGENASE

### FIELD OF THE INVENTION

The present invention relates to a lipoxygenase and a polynucleotide encoding it.

### 5 BACKGROUND OF THE INVENTION

Lipoxygenase (EC 1.13.11.12) is an enzyme that catalyzes the oxygenation of poly-unsaturated fatty acids such as linoleic acid, linolenic acid and arachidonic acid, which contain a *cis,cis*-1,4-pentadiene unit and produces hydroperoxides of these fatty acids. The enzyme is widely distributed in plants and animals. A number of lipoxygenase genes have been isolated  
10 from various plant and mammalian sources.

On the other hand, only a limited number of microbial lipoxygenases are known, and no lipoxygenase gene of microbial origin has been described. Su and Oliw, J. Biological Chemistry, 273 (21), 13072-79 (1998) describe a lipoxygenase from *Gaeumannomyces graminis*.  
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### SUMMARY OF THE INVENTION

The inventors have found a novel fungal lipoxygenase and determined its sequence, which can be used for the production of the enzyme in industrial scale. They have cloned the gene into *E. coli* and deposited the clone.

20 Accordingly, the invention provides a lipoxygenase which is:

- a) a polypeptide encoded by a DNA sequence cloned into plasmid pUC19 present in *Escherichia coli* deposited as DSM 14139,
- b) a polypeptide having an amino acid sequence as the mature peptide shown in SEQ ID NO: 1, or which can be obtained therefrom by substitution, deletion, and/or insertion  
25 of one or more amino acids,
- c) an analogue of the polypeptide defined in (a) or (b) which:
  - i) has at least 50 % homology with said polypeptide,
  - ii) is immunologically reactive with an antibody raised against said polypeptide in purified form,
  - 30 iii) is an allelic variant of said polypeptide, or
- d) a polypeptide encoded by DNA that hybridizes under low stringency conditions with a complementary strand of
  - i) the DNA sequence cloned into plasmid pUC19 present in *Escherichia coli* deposited as DSM 14139 or
  - 35 ii) the DNA sequence of SEQ ID NO: 1 encoding the mature polypeptide or a subsequence thereof having at least 100 nucleotides.

The invention also provides a polynucleotide which comprises:

a) the partial DNA sequence encoding a mature lipoxygenase cloned into a plasmid present in *Escherichia coli* DSM 14139,

b) the partial DNA sequence encoding a mature lipoxygenase shown in SEQ ID NO: 1,

c) an analogue of the sequence defined in a) or b) which encodes a lipoxygenase and

i) has at least 60 % homology with said DNA sequence, or

ii) hybridizes at high stringency with a complementary strand of said DNA sequence or a subsequence thereof having at least 100 nucleotides,

iii) is an allelic variant thereof, or

d) a complementary strand of a), b) or c).

Other aspects of the invention provide a nucleic acid construct and a recombinant expression vector comprising the polynucleotide, a recombinant host cell comprising the construct or the vector, and a method of producing a lipoxygenase by cultivating the cell. Further, the invention provides a method of screening a eukaryotic library to obtain a lipoxygenase and an oligonucleotides probe useful for screening. Finally, the invention provides use of the lipoxygenase in baking and in a detergent.

## DETAILED DESCRIPTION OF THE INVENTION

### Genomic DNA source

A lipoxygenase gene of the invention may be derived from a filamentous fungus, e.g. an Ascomycota, particularly Magnaporthaceae, such as a strain of *Magnaporthe*, particularly *Magnaporthe salvinii* Cattaneo (*Mycologia* 64 (1), 110 (1972)). The species is also known under the synonyms *Curvularia sigmoidea*, *Helminthosporium sigmoideum*, *Leptosphaeria salvinii*, *Nakataea sigmoidea*, *Sclerotium oryzae* and *Vakrabeeja sigmoidea*. An example is the strain *M. salvinii* IFO 6642.

Alternatively, the gene may be isolated from *Pyricularia*, e.g. *P. oryzae* or *P. grisea*, e.g. *P. oryzae* IFO 30517. The IFO strains are available on commercial terms from Institute for Fermentation, Osaka (IFO), 17-85, Juso-honmachi 2-chome, Yodogawa-ku, Osaka 532-8686, Japan.

The lipoxygenase gene may be isolated from these organisms using probes designed on the basis of the DNA sequences in this specification.

A strain of *Escherichia coli* containing a lipoxygenase gene from *M. salvinii* IFO 6642 was deposited by the inventors under the terms of the Budapest Treaty with the DSMZ - Deutsche Sammlung von Microorganismen und Zellkulturen GmbH, Mascheroder Weg 1b, D-38124 Braunschweig DE, Germany. The deposit date was 28 February 2001, and the accession number was DSM 14139.

## Production of lipoxygenase by cultivation of transformant

The lipoxygenase of the invention may be produced by transforming a suitable host cell with a DNA sequence encoding the lipoxygenase, cultivating the transformed organism under conditions permitting the production of the enzyme, and recovering the enzyme from the culture.

The host organism may be a eukaryotic cell, in particular a fungal cell, such as a yeast cell or a filamentous fungal cell, e.g. a strain of *Aspergillus*, *Fusarium*, *Trichoderma* or *Saccharomyces*, particularly *A. niger*, *A. oryzae*, *F. graminearum*, *F. sambucinum*, *F. cerealis* or *S. cerevisiae*. The production of the lipoxygenase in such host organisms may be done by the general methods described in EP 238,023 (Novo Nordisk), WO 96/00787 (Novo Nordisk) or EP 244,234 (Alko).

## Properties of LOX

The lipoxygenase of the invention is able to oxidize a wide range of substrates containing a *cis-cis*-pentadienyl moiety. Thus, it acts on polyunsaturated fatty acids such as linoleic acid (18 carbon atoms, 2 double bonds), linolenic acid (18:3), arachidonic acid (20:4), eicosapentaenoic acid (EPA, 20:5) and docosahexaenoic acid (DHA, 22:6). It also acts on substrates other than fatty acids, such as methyl linoleate and probably also triglycerides. The enzyme has a very low Michaelis constant ( $K_M$ ) for linoleic acid and a high specificity ( $V_{max}/K_M$ ) towards this substrate.

The lipoxygenase from *M. salvinii* is a 9-lipoxygenase, i.e. it oxidizes the double bond between carbon atoms 9 and 10 in linoleic acid and linolenic acid.

The lipoxygenase from *M. salvinii* has optimum activity around pH 7, and it is highly active over a broad pH range 3-12, having more than 50 % of optimum activity in the range pH 6-11. It is stable after overnight incubation at pH 5-11.

The native lipoxygenase from *M. salvinii* has optimum activity at 50-60°C. It is quite active at 40-60°C, and the activity begins to decline at 70 °C. The lipoxygenase is stable after 30 minutes incubation at pH 7 at temperatures up to 50°C.

The reaction rate for recombinant lipoxygenase (expressed in *A. oryzae*) increases nearly ten times at the optimal temperature for catalysis compared to the rate obtained at room temperature. The maximum reaction rate is obtained at 67.5°C. A steep decrease in rate constant is seen above the temperature optimum. It is believed that glycosylation renders the recombinant enzyme more stable towards heat than the wild-type enzyme.

The recombinant lipoxygenase is quite stable at temperatures up to 50°C for at least one hour. The activity drops in a linear fashion at higher temperatures between 50-60°C, and no activity is detected after incubations above 60°C for one hour. No activity loss is detected during incubation at temperatures below 45°C.

Frozen solutions of the lipoxygenase lose some activity during storage. With addition of 10 % glycerol there is no discernible activity loss after two weeks storage at -20°C, and the enzyme survived repeated cycles of thaw-freeze without loss of activity.

The lipoxygenase of the invention has good stability in the presence of anionic surfactants. Thus, the lipoxygenase from *M. salvinii* is stable in the presence of 400 ppm of LAS (linear alkyl-benzene sulfonate).

### Use of lipoxygenase

The lipoxygenase can be used for green flavor synthesis, e.g. nonenal from 9-hydroperoxide of linolenic acid. The synthesis may be done in analogy with Whitehead et al.1995, Cereal foods world 40(4), 193-197 and US 4769243.

The lipoxygenase can also be used for plant hormone synthesis as described in JP H11-29410.

Also the lipoxygenase is a good oxidant of carotenoids, so it can be used for bleaching of foodstuffs such as flour, oil or marine food including carotenoids or carotenoid-like pigments.

The oxidation activity can be utilized for cross-linking of protein, oil, starch, fiber and mixture of these. Cross-linking of chemical compounds can be utilized for synthesis of polymer to give plastic fiber or plastic resin. It can be used for bleaching as a detergent for phenolic, carotenoid or fatty stains or dinginess. Or it can be used for bleaching of waste water or textile dye.

Lipoxygenase can be used for bleaching of plant or marine food materials containing of carotenoids. Thus it could be used for bleaching of flour for bread, noodle or pasta, or bleaching of fish meat or fish oil containing astaxanthin.

It also can be used for cross-linking of protein, oil, starch, plant-fiber or mixture of these in presence of fatty acid, oil or fats. It is useful to change the texture or physical properties of foodstuff or to control of flavor for fat and oil, or to produce polymers made of natural stuff beside food use. Cross-linked compounds can be chemical compounds, e.g. phenolic, carbonyl, carboxyl or amide compounds or mixture of these. It could be used for synthesis of plastic fiber or resin.

Other usages of lipoxygenase can be the synthesis of flavor compound such as hexanal or hexenal together as synergy effect of hydroperoxide lyase. Or in case plant material is used as the source of above two enzymes, lipoxygenase can be added to it to improve the yield of flavor compound. The similar can be done for synthesis of plant or animal hormones.

Finally it can be used as bleaching agent. It can be used in detergents for bleaching of phenolic, carotenoid, fatty stains or dinginess of clothes. Or it can be used for bleaching of textile dye or dye for pulp industry in waste water or changing of dye texture.

**Recombinant expression vector**

The expression vector of the invention typically includes control sequences encoding a promoter, operator, ribosome binding site, translation initiation signal, and, optionally, a selectable marker, a transcription terminator, a repressor gene or various activator genes. The vector may be an autonomously replicating vector, or it may be integrated into the host cell genome.

**Production by cultivation of transformant**

The lipoxxygenase of the invention may be produced by transforming a suitable host cell with a DNA sequence encoding the lipoxxygenase, cultivating the transformed organism under conditions permitting the production of the enzyme, and recovering the enzyme from the culture.

The host organism may be a eukaryotic cell, in particular a fungal cell, such as a yeast cell or a filamentous fungal cell, e.g. a strain of *Aspergillus*, *Fusarium*, *Trichoderma* or *Saccharomyces*, particularly *A. niger*, *A. oryzae*, *F. graminearum*, *F. sambucinum*, *F. cerealis* or *S. cerevisiae*. The production of the lipoxxygenase in such host organisms may be done by the general methods described in EP 238,023 (Novo Nordisk), WO 96/00787 (Novo Nordisk) or EP 244,234 (Alko).

The enzyme can be purified in one step by cation-exchange chromatography to homogeneity.

**Nucleotide probe**

A nucleotide probe may be designed on the basis of the DNA sequence of SEQ ID NO: 1 or the polypeptide sequence of SEQ ID NO: 2, particularly the mature peptide part. The probe may be used in screening for LOX-encoding DNA as described below.

A synthetic oligonucleotide primer may be prepared by standard techniques (e.g. as described in Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual (2<sup>nd</sup> edn.) Cold Spring Harbor Laboratory, Cold Spring Harbor, New York) on the basis of the mature part of the amino acid sequence in SEQ ID NO: 2 or the corresponding part of the DNA sequence. It may be a degenerate probe and will typically contain at least 20 nucleotides.

**Screening of eukaryotic DNA library**

A polypeptide with lipoxxygenase activity may be obtained by a method comprising:  
a) preparing a eukaryotic DNA library,  
b) screening the library to select DNA molecules which hybridize to the probe described above,

- c) transforming host cells with the selected DNA molecules,
- d) cultivating the transformed host cells to express polypeptides encoded by the DNA molecules, and
- e) assaying the expressed polypeptides to select polypeptides having lipoxxygenase activity.

The eukaryotic DNA library may be prepared by conventional methods. It may include genomic DNA or double-stranded cDNA derived from suitable sources such as those described above.

Molecular screening for DNA sequences may be carried out by polymerase chain reaction (PCR) followed by hybridization.

In accordance with well-known procedures, the PCR fragment generated in the molecular screening may be isolated and subcloned into a suitable vector. The PCR fragment may be used for screening DNA libraries by e.g. colony or plaque hybridization.

## Hybridization

The hybridization is used to indicate that a given DNA sequence is analogous to a nucleotide probe corresponding to a DNA sequence of the invention. The hybridization may be done at low, medium or high stringency. One example of hybridization conditions is described in detail below.

Suitable conditions for determining hybridization between a nucleotide probe and a homologous DNA or RNA sequence involves presoaking of the filter containing the DNA fragments or RNA in 5 x SSC (standard saline citrate) for 10 min, and prehybridization of the filter in a solution of 5 x SSC (Sambrook et al. 1989), 5 x Denhardt's solution (Sambrook et al. 1989), 0.5 % SDS and 100 µg/ml of denatured sonicated salmon sperm DNA (Sambrook et al. 1989), followed by hybridization in the same solution containing a random-primed (Feinberg, A. P. and Vogelstein, B. (1983) *Anal. Biochem.* 132:6-13), <sup>32</sup>P-dCTP-labeled (specific activity > 1 x 10<sup>9</sup> cpm/µg ) probe for 12 hours at approx. 45°C. The filter is then washed two times for 30 minutes in 2 x SSC, 0.5 % SDS at a temperature of at least 55°C, particularly at least 60°C, more particularly at least 65°C, e.g. at least 70°C, or at least 75°C.

Molecules to which the oligonucleotide probe hybridizes under these conditions are detected using an x-ray film.

## Alignment and homology

The lipoxxygenase and the nucleotide sequence of the invention may have homologies to the disclosed sequences of at least 75 % or at least 85 %, particularly at least 90 % or at least 95 %, e.g. at least 98 %.

For purposes of the present invention, alignments of sequences and calculation of

homology scores were done using a Needleman-Wunsch alignment (i.e. global alignment), useful for both protein and DNA alignments. The default scoring matrices BLOSUM50 and the identity matrix are used for protein and DNA alignments respectively. The penalty for the first residue in a gap is -12 for proteins and -16 for DNA, while the penalty for additional residues in a gap is -2 for proteins and -4 for DNA. Alignment is from the FASTA package version v20u6 (W. R. Pearson and D. J. Lipman (1988), "Improved Tools for Biological Sequence Analysis", PNAS 85:2444-2448, and W. R. Pearson (1990) "Rapid and Sensitive Sequence Comparison with FASTP and FASTA", Methods in Enzymology, 183:63-98).

## EXAMPLES

### Materials and Methods

Molecular cloning techniques are described in Sambrook et al. (1989).

The following commercial plasmids and *E. coli* strains were used for sub-cloning and DNA library construction:

pT7Blue (Novagen)

pUC19 (TOYOBO, Japan)

*E. coli* JM109 (TOYOBO, Japan)

*E. coli* DH12S (GIBCO BRL, Life Technologies, USA)

Labeling and detection of hybridization probe was done using DIG-labeling and detection Kit (Boehringer Mannheim). Nylon membrane Hybond-N+ (Amersham, England) was used for DNA transfer for colony hybridization.

Soybean lipoxygenase (type I-B) (cat.# L7315) and astaxanthin (cat.# A-9335) was supplied by Sigma. b-carotene (cat.# 031-05533) were supplied by Wako.

### Media and buffer solution

COVE-ar: per liter 342.3 g sucrose, 20 ml COVE salt solution, 10 mM acryl amide, 15 mM CsCl<sub>2</sub>, 30 g Agar noble (Difco)

COVE2-ar: per liter 30 g sucrose, 20 ml COVE salt solution, 10 mM acrylamide, 30 g Agar noble (Difco)

COVE salt solution: per liter 26 g KCl, 26 g MgSO<sub>4</sub>-7H<sub>2</sub>O, 76 g KH<sub>2</sub>PO<sub>4</sub>, 50ml Cove trace metals.

Cove trace metals: per liter 0.04 g NaB<sub>4</sub>O<sub>7</sub>-10H<sub>2</sub>O, 0.4 g CuSO<sub>4</sub>-5H<sub>2</sub>O, 1.2 g FeSO<sub>4</sub>-7H<sub>2</sub>O, 0.7 g MnSO<sub>4</sub>-H<sub>2</sub>O, 0.7 g Na<sub>2</sub>MoO<sub>2</sub>-2H<sub>2</sub>O, 0.7 g ZnSO<sub>4</sub>-7H<sub>2</sub>O.

AMG trace metals: per liter 14.3 g ZnSO<sub>4</sub>-7H<sub>2</sub>O, 2.5 g CuSO<sub>4</sub>-5H<sub>2</sub>O, 0.5 g NiCl<sub>2</sub>, 13.8 g FeSO<sub>4</sub>, 8.5 g MnSO<sub>4</sub>, 3.0 g citric acid.

YPG: per liter 4 g yeast extract, 1 g KH<sub>2</sub>PO<sub>4</sub>, 0.5 g MgSO<sub>4</sub>-7H<sub>2</sub>O, 15 g glucose, pH 6.0.



STC: 0.8 M Sorbitol, 25 mM Tris pH 8, 25 mM CaCl<sub>2</sub>.

STPC: 40% PEG4000 in STC buffer.

Cove top agarose: per liter 342.3 g sucrose, 20 ml COVE salt solution, 10 mM Acetamide, 10 g low melt agarose.

5 MS-9: per liter 30 g soybean powder, 20 g glycerol, pH 6.0.

MDU-2Bp: per liter 45 g maltose-1H<sub>2</sub>O, 7 g yeast extract, 12 g KH<sub>2</sub>PO<sub>4</sub>, 1 g MgSO<sub>4</sub>-7H<sub>2</sub>O, 2 g K<sub>2</sub>SO<sub>4</sub>, 5 g Urea, 1 g NaCl, 0.5 ml AMG trace metal solution pH 5.0.

#### Host organism

10 *Aspergillus oryzae* BECh2 is described in WO 00/39322. It is a mutant of JaL228 (described in WO98/123000), which is a mutant of IFO4177.

#### Transformation of *A. oryzae*

*Aspergillus oryzae* strain BECh2 was inoculated in 100 ml of YPG medium and incubated at 32°C for 16 hours with stirring at 80 rpm. Grown mycelia was collected by filtration followed by washing with 0.6 M KCl and re-suspended in 30 ml of 0.6 M KCl containing Glucanex® (Novozymes) at the concentration of 30 µl/ml. The mixture was incubated at 32°C with the agitation at 60 rpm until protoplasts were formed. After filtration to remove the remained mycelia, protoplasts were collected by centrifugation and washed with STC buffer twice. The protoplasts were counted with a hematology and re-suspended in a solution of STC:STPC:DMSO (8:2:0.1) to a final concentration of 1.2 x 10<sup>7</sup> protoplasts/ml. About 4 µg of DNA was added to 100 µl of protoplast solution, mixed gently and incubated on ice for 30 minutes. 1 µl STPC buffer was added to the mixture and incubated at 37°C for another 30 minutes. After the addition of 10 ml of Cove top agarose pre-warmed at 50°C, the reaction mixture was poured onto COVE-ar agar plates. The plates were incubated at 32°C for 5 days.

#### SDS-PAGE

SDS polyacrylamide electrophoresis was carried out using the commercialized gel PAGEL AE6000 NPU-7.5L (7.5T%) with the apparatus AE-6400 (Atto, Japan) following the provided protocol. 15 µl of sample was suspended in 15 µl of 2x conc. of sample loading buffer (100 mM Tris-HCl (pH 6.8), 200 mM Dithiothreitol, 4% SDS, 0.2% Bromophenol blue and 20% glycerol) and boiled for 5 minutes. 20 µl of sample solution was applied to a polyacrylamide gel, and subjected for electrophoresis in the running buffer (25 mM Tris, 0.1% SDS, 192 mM Glycine) at 20 mA per gel. Resulting gel was stained with SYPRO Orange and detected by molecular Imager FX (BIO-RAD).

Assays for lipoxygenase activitySpectrophotometric assay

Lipoxygenase activity was determined spectrophotometrically at 25°C by following the formation of hydroperoxides with the absorbance at 234 nm. To 0.98 ml of the buffer (50 mM  $\text{KH}_2\text{PO}_4/\text{NaHPO}_4$ , pH 7.0), 10  $\mu\text{l}$  of substrate solution (10mM linolenic acid dispersed with 0.2% Tween20) was added and the reaction was started by the addition of 10  $\mu\text{l}$  of enzyme solution. One unit causes an increase in absorbance at 234 nm of 0.001/min.

FOX assay

The assay was initiated by the addition of 20  $\mu\text{l}$  enzyme solution to 80  $\mu\text{l}$  of 50 mM each buffer containing 0.7 mM linolenic acid dispersed with 0.02% of Tween 20 using Hisco-tron, and incubated for 10 min. The assay was terminated by the addition of 900  $\mu\text{l}$  of FOX reagent: sulfuric acid (25 mM), xylanol orange(100  $\mu\text{M}$ ), iron(II) sulfate (100  $\mu\text{M}$ ), butylated hydroxytoluen (4 mM) in methanol:water (9:1). Blanks contained only substrate solution during the incubation, but enzyme solution was added after the addition of FOX reagent. The yellow color of acidified xylanol orange was converted to a blue color by the lipid hydroperoxide-mediated oxidation of  $\text{Fe}^{2+}$  ions with the dye. Absorbance of the  $\text{Fe}^{3+}$  complex at 620 nm was measured 1 hour after the addition of FOX reagent.

Bleaching assay

Bleaching effect by lipoxygenase was examined spectrophotometrically at 25°C by following the absorbance at 470 nm. The pigment solution was prepared as follows. 150  $\mu\text{l}$  of stock pigment solution (1mg each pigment in 1ml chloroform) was evaporated to be dry. Then 30 ml of the buffer (50 mM  $\text{KH}_2\text{PO}_4/\text{NaHPO}_4$ , pH 7.0) with 0.3% of Tween 20 was added slowly and the pigment was dissolved. To 0.98 ml of the pigment solution, 10  $\mu\text{l}$  of substrate solution (10mM linolenic acid dispersed with 0.2% of Tween20) was added and the reaction was started by the addition of 10  $\mu\text{l}$  of enzyme solution.

**Example 1: Cloning of genomic LOX gene from *M. salvinii***

Genomic DNA from *Magnaporthe salvinii* was digested with *Sac I* and separated on 1.0% agarose gel. Around 2.5 kbp of DNA digestion was recovered from the gel and ligated with BAP treated pUC19 linearized by *Sac I*. Ligation mixture was transformed into *E. coli* DH12S to construct a partial genomic library. It was screened, and a lipoxygenase-positive *E. coli* colony was isolated and the plasmid, termed pSG28, was recovered. The plasmid pSG28 contained a 2.5 kbp *SacI* genomic fragment that contained the presumed LOX homologue sequence. The sequence of 1973 bp out of 2.5 kbp is shown as SEQ. ID 1.

Introns were identified and are indicated in SEQ ID NO: 1. The splice sites were pre-

dicted as described in S.M. Hebsgaard et al., Nucleic Acids Research, 1996, Vol. 24, No. 17, 3439-3452.

The presumed open reading frame consisted of 1851 bp, and the deduced amino acid sequence corresponded to 617 amino acids, shown as SEQ ID NO: 2. The molecular mass was estimated as 67500 Da.

The *E. coli* DH12S harboring plasmid pSG28 was deposited at DSMZ as DSM 14139 with the accession date 2001-02-28.

## Example 2: Expression of *M. salvinii* LOX in *A. oryzae*

### Construction of expression plasmid

The partial genomic sequence of *M. salvinii* genomic gene was amplified by PCR using pSG28 as a template. Primer 3 and 4 (SEQ ID NO: 3 and 4) were designed to make *Bam*H I and *Xho* I sites at both ends of the PCR product (nucleotides 4-9 of primer 3 and 5-10 of primer 4, respectively). PCR reaction mixture comprised of 2.5 mM dNTP, 30 pmol each of primer 3 and 4, 5 units of LA taq polymerase (Takara) and supplied GC buffer I. Reaction condition was shown below. LA taq polymerase was added to the reaction mixture after step 1.

Step	Temperature	Time
1	98 °C	10 mins
2	96 °C	20 sec
3	55 °C	45 sec
4	72 °C	30 sec
5	72 °C	10 mins

\* Step 2 to Step 4 were repeated 30 times.

PCR amplified 1.9 kb fragment was isolated and cloned into pT7Blue resulting in pSG29.

The plasmid pSG29 was digested by *Bam*HII and *Xho*I and 1.9 kb of fragment which contained the LOX gene was ligated with pMT2188 digested with *Bam*HI and *Xho*I. The plasmid pMT2188 has a modified *Aspergillus niger* neutral amylase promoter, *Aspergillus nidulans* TPI leader sequence, *Aspergillus niger* glucoamylase terminator, *Aspergillus nidulans* amdS gene as a marker for fungal transformation and *S. cerevisiae* ura3 as the marker for *E. coli* transformation. Transformation was done with *E. coli* DB6507 in which pyrF gene is deficient and can be complemented with *S. cerevisiae* Ura3. Resulting plasmid was termed pSG30.

Expression of *M. salvinii* LOX in *A. oryzae*

*A. oryzae* BECh2 was transformed with the plasmid pSG30 and selection positive transformants were isolated. Transformants were grown on COVE 2 -ar at 32°C for 5 days and inoculated to 100 ml of MS-9 shaking flask. After the cultivation with vigorous agitation at 32°C for 1 day, 3 ml of each culture was transferred to 100 ml of MDU-2Bp in shaking flask to cultivate at 32°C for 3 days. Culture broth was centrifuged at 3500 rpm for 10 minutes and supernatant was collected.

Lipoxygenase activities of the supernatant were determined spectrophotometrically as described before. Positive transformants showed about 100,000U/ml culture broth while untransformed *A. oryzae* BECh2 showed no activity. Culture supernatant was also subjected to SDS-PAGE analysis. Positive transformants showed 80-100kDa smear band which indicated the protein was heavily glycosylated. Untransformed *A. oryzae* BECh2 did not show any significant bands.

**Example 3: Substrate specificity of lipoxygenase**

Kinetic parameters for a number of substrates were determined by standard methods for the *M. salvinii* lipoxygenase.

Substrate	$V_{\max}$ ( $\mu\text{mol}/\text{min}/\text{mg}$ )	$K_M$ ( $\mu\text{M}$ )	$V_{\max}/K_M$ ( $\mu\text{mol}/\text{min}/\text{mg}/\mu\text{M}$ )
Linoleic acid	2.63	1	2.557
Na linoleate	2.07	0.41	5.061
Linoelaidic acid	No activity	No activity	No activity
Linolenic acid	1.9	0.4	4.488
Eicosadienoic acid	2.02	11	0.177
Arachidonic acid	2.44	5.5	0.446
Linoleoyl chloride	0.97	12	0.080
Methyl linoleate	0.82	30	0.026
Linoleoyl acetate	0.77	9	0.085
Linoleoyl alcohol	1.4	8	0.175

For comparison, one substrate was also tested with soybean lipoxygenase.

Substrate	$V_{\max}$ ( $\mu\text{mol}/\text{min}/\text{mg}$ )	$K_M$ ( $\mu\text{M}$ )	$V_{\max}/K_M$ ( $\mu\text{mol}/\text{min}/\text{mg}/\mu\text{M}$ )
Linoleic acid	12.3	230	0.054

### Example 3: pH dependence of lipoxygenase activity

- 5 The relative activity of the *M. salvinii* lipoxygenase at various pH values was determined by the FOX assay described above, using the following buffers: 50 mM citric acid/sodium citrate (pH 2.21-3.73),  $\text{KH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$  (pH 5.30,6.17), Tris/HCl (pH 7.01,8.02), glycylglycine NaCl/NaOH (pH 9.33-11.0).

pH	Relative Activity (%)
2.21	7.11
2.90	20.6
3.73	27.7
5.30	60.0
6.17	83.7
7.01	100
8.02	92.9
9.33	82.6
11.0	77.7

10

### Example 4: Temperature dependence of lipoxygenase activity

The effect of temperature on the *M. salvinii* lipoxygenase was studied by 10 min incubation at pH 7.0.

15

Temperature	Relative Activity (%)
25	50.1
40	90.0
50	100
60	99.6
70	60.4

**Example 5: Bleaching effect of lipoxygenases**

The bleaching effect of *M. salvinii* LOX was examined. Soybean L1 was included for comparison.  $\beta$ -carotene and astaxanthin were used as pigments.

$\beta$ -carotene	Time (min)	<i>M. salvinii</i>	Soybean L1
	0	0.3783	0.3575
	0.4	0.3791	0.3616
	0.8	0.3729	0.3601
	1.2	0.3702	0.362
	1.4	0.3685	0.3602
	1.8	0.3651	0.3602
	2.2	0.3633	0.3595
	2.6	0.3486	0.3595
	3	0.341	0.3594
$\Delta A_{470}/\text{min}$		0.0121	0.00005
LOX activity		2.652	1.962

5

Astaxanthin	Time (min.)	<i>M. salvinii</i>	Soybean L1
	0	0.5292	0.5026
	0.4	0.5244	0.5029
	0.8	0.5177	0.505
	1	0.5166	0.5025
	1.4	0.512	0.5013
	1.8	0.5004	0.4993
	2.2	0.4876	0.4985
	2.6	0.4714	0.4986


	3	0.4566	0.498
$\Delta A_{470}/\text{min}$		0.0239	0.0021
<b>LOX activity</b>		2.4952	2.018

The results show that *M. salvinii* LOX bleaches the pigment solutions. Soybean LOX showed little effect on bleaching.

0-1	Form - PCT/RO/134 (EASY) Indications Relating to Deposited Microorganism(s) or Other Biological Material (PCT Rule 13bis)	
0-1-1	Prepared using	PCT-EASY Version 2.92 (updated 01.01.2002)
0-2	International Application No.	
0-3	Applicant's or agent's file reference	10148

1	The indications made below relate to the deposited microorganism(s) or other biological material referred to in the description on:	
1-1	page	2
1-2	line	36-37
1-3	Identification of Deposit	
1-3-1	Name of depositary institution	DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
1-3-2	Address of depositary institution	Mascheroder Weg 1b, D-38124 Braunschweig, Germany
1-3-3	Date of deposit	28 February 2001 (28.02.2001)
1-3-4	Accession Number	DSMZ 14139
1-4	Additional Indications	NONE
1-5	Designated States for Which Indications are Made	all designated States
1-6	Separate Furnishing of Indications These indications will be submitted to the International Bureau later	NONE

## FOR RECEIVING OFFICE USE ONLY

0-4	This form was received with the international application: (yes or no)	YES
0-4-1	Authorized officer	 Marie Louise Rosendal Head Clerk

## FOR INTERNATIONAL BUREAU USE ONLY

0-5	This form was received by the international Bureau on:	
0-5-1	Authorized officer	



**CLAIMS**

1. A lipoxygenase which is:

a) a polypeptide encoded by a DNA sequence cloned into plasmid pUC19 present in *Escherichia coli* deposited as DSM 14139,

b) a polypeptide having an amino acid sequence as the mature peptide shown in SEQ ID NO: 1, or which can be obtained therefrom by substitution, deletion, and/or insertion of one or more amino acids,

c) an analogue of the polypeptide defined in (a) or (b) which:

i) has at least 50 % homology with said polypeptide,

ii) is immunologically reactive with an antibody raised against said polypeptide in purified form,

iii) is an allelic variant of said polypeptide, or

d) a polypeptide encoded by DNA that hybridizes under low stringency conditions with a complementary strand of

i) the DNA sequence cloned into plasmid pUC19 present in *Escherichia coli* deposited as DSM 14139 or

ii) the DNA sequence of SEQ ID NO: 1 encoding the mature polypeptide or a subsequence thereof having at least 100 nucleotides.

2. The lipoxygenase of claim 1 which is derived from a filamentous fungus, e.g. an Ascomycota, such as a strain of *Magnaporthe*, particularly *M. salvinii*, more particularly strain IFO 6642.

3. DNA comprising a nucleic acid sequence which encodes the lipoxygenase of claim 1 or 2.

4. A polynucleotide which comprises:

a) the partial DNA sequence encoding a mature lipoxygenase cloned into a plasmid present in *Escherichia coli* DSM 14139,

b) the partial DNA sequence encoding a mature lipoxygenase shown in SEQ ID NO: 1,

c) an analogue of the sequence defined in a) or b) which encodes a lipoxygenase and

i) has at least 60 % homology with said DNA sequence, or

ii) hybridizes at high stringency with a complementary strand of said DNA sequence or a subsequence thereof having at least 100 nucleotides,

iii) is an allelic variant thereof, or

d) a complementary strand of a), b) or c).

5. A nucleic acid construct comprising the nucleic acid sequence of claim 3 or 4 operably linked to one or more control sequences capable of directing the expression of the lipoxxygenase in a suitable expression host.

5 6. A recombinant expression vector comprising the nucleic acid construct of claim 5, a promoter, and transcriptional and translational stop signals.

7. A recombinant host cell comprising the nucleic acid construct of claim 5 or the vector of claim 6.

10

8. A method for producing a lipoxxygenase comprising cultivating the host cell of claim 7 under conditions conducive to production of the lipoxxygenase, and recovering the lipoxxygenase.

15 9. An oligonucleotide probe which consists of at least 20 nucleotides and which encodes a partial polypeptide sequence of SEQ ID NO: 2.

10. A method for obtaining a polypeptide with lipoxxygenase activity, comprising:

- 20 a) preparing a eukaryotic DNA library,  
b) screening the library to select DNA molecules which hybridize to the probe of claim 9,  
c) transforming host cells with the selected DNA molecules,  
d) cultivating the transformed host cells to express polypeptides encoded by the DNA molecules, and  
e) assaying the expressed polypeptides to select polypeptides having lipoxxygenase activity.
- 25

11. A method for preparing a dough or a baked product made from dough, comprising adding the lipoxxygenase of claim 1 or 2 to the dough.

30 12. A dough composition comprising the lipoxxygenase of claim 1 or 2.

13. A detergent composition comprising a surfactant and the lipoxxygenase of claim 1 or 2.

14. The detergent composition of the preceding claim wherein the surfactant is anionic.

35

15. A process for oxidizing a polyunsaturated fatty acid comprising contacting the acid with the lipoxxygenase of claim 1 or 2 in the presence of air.

16. Use of the process of the preceding claim for green flavor synthesis or plant hormone synthesis.

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SEQUENCE LISTING

<110> Novozymes A/S

<120> Lipoxygenase

<130> 10148-WO

<160> 4

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## 10148-WO.ST25

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## 10148-WO.ST25

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## INTERNATIONAL SEARCH REPORT

International Application No

PCT/DK 02/00251

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N9/02 A21D8/04 C12P7/64 C12N15/63 C11D3/386

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N A21D C12P C11D

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

PAJ, EPO-Internal, SEQUENCE SEARCH

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 02 20730 A (NOVOZYMES AS) 14 March 2002 (2002-03-14) claims 1-17 & DATABASE EBI [Online] retrieved from EBI Database accession no. ax398040 77% identity in 618 aa overlap with SEQ ID No.2	1-8, 10-16
A	WO 00 60093 A (INST PFLANZENBIOCHEMIE IPB (DE)) 12 October 2000 (2000-10-12) claims 1-14 --- -/--	1-8, 10-16



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

\* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&amp;" document member of the same patent family

Date of the actual completion of the international search

1 August 2002

Date of mailing of the international search report

15. 08. 2002

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
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Fax: (+31-70) 340-3016

Authorized officer

ekström nils

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/DK 02/00251

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	PATENT ABSTRACTS OF JAPAN vol. 2000, no. 07, 29 September 2000 (2000-09-29) & JP 2000 106832 A (HONEN CORP), 18 April 2000 (2000-04-18) abstract; claims 1-5 ---	1-8, 10-16
A	US 6 204 037 B1 (BRASH A R ET AL) 20 March 2001 (2001-03-20) claims 1-22 ---	1-8, 10-16
P,A	WO 01 29227 A (BASF AG) 26 April 2001 (2001-04-26) claims 1-20 ---	1-8, 10-16
P,A	WO 01 79560 A (GLAXO GROUP LTD ;EDWARDS LISA D (US); EMMETT AMANDA H (US); ANDERS) 25 October 2001 (2001-10-25) claims 1-31 ---	1-8, 10-16
P,A	WO 01 90323 A (MILLENNIUM PHARM INC (US)) 29 November 2001 (2001-11-29) claims 1-22 ---	1-8, 10-16
P,A	HÖRNSTEN L ET AL: "Cloning of the manganese lipxygenase gene reveals homology with the lipxygenase gene family." EUR. J. BIOCHEM., vol. 269, 2002, pages 2690-2697, XP002902589 the whole document -----	1-8, 10-16

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/DK 02/00251

### Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☒ Claims Nos.: 9  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:  
see FURTHER INFORMATION sheet PCT/ISA/210
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

### Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

#### Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 9

Claim 9 relates to oligonucleotid consisting of at least 20 nucleotides and encodes a partial polypeptide of SEQ. ID. No. 2. These polypeptides are 601 amino acids long- encoded by more than 1800 nucleotides; claim 9 covers a large number of possibilities. In view of the large number of possibilities- not limited even by a general functionality- render it difficult, if not impossible, to determine the matter for which protection is sought. The present claim fails to comply with the clarity and conciseness requirements of Article 6 PCT to such extent that a meaningful search is impossible. As a consequence of this, claim 9 has not been searched.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/DK 02/00251

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